JOURNAL OF CLINICAL MICROBIOLOGY, May 1987, p. 796–801
0095-1137/87/050796-06$02.00/0

Vol. 25, No. 5

Crohn's Disease-Isolated Mycobacteria Are Identical to *Mycobacterium paratuberculosis*, as Determined by DNA Probes That Distinguish between Mycobacterial Species

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Received 15 July 1986/Accepted 15 January 1987

DNA extracted from an unclassified Crohn's disease-isolated *Mycobacterium* strain was cloned. The recombinant clones were radiolabeled and hybridized to restriction digests of mycobacterial DNA transferred to nylon membranes. Restriction fragment length polymorphisms (RFLPs) were identified that distinguished between mycobacterial DNA samples. Quantitative estimates of frequencies of DNA base substitution were also obtained. No RFLPs were detected between the DNA of three unclassified Crohn's disease-isolated mycobacteria and *Mycobacterium paratuberculosis*, although several RFLPs were detected that distinguished between *M. paratuberculosis* and both *M. avium* complex serovars 2 and 5. The frequency of DNA base substitution between *M. paratuberculosis* and *M. avium* complex serovar 2 was measured as 0.87 (±1.2)%.

Crohn's disease (CD) is a chronic granulomatous inflammation principally of the large and small intestine, but lesions may also be found in any part of the alimentary tract as well as in the skin, liver, and joints. Its resemblance to intestinal tuberculosis initially suggested mycobacterial involvement (13), but the failure to cultivate specific organisms from CD tissue and the inability to detect acid-fast bacilli led to a retreat from this concept. However, the possibility that mycobacteria are causative agents of CD has recently been revived by the independent isolation of mycobacteria from CD tissue by a number of groups. Chiodini et al. (7) described the isolation of a slow-growing, mycobactin-dependent *Mycobacterium* species from the intestinal mucosa of CD patients but not from control tissue. When goats were orally inoculated with this organism, the development of CD-like lesions was observed in the intestines. The biochemical and culture characteristics of this *Mycobacterium* sp. were similar to those of *Mycobacterium paratuberculosis*, the causative agent of Johne's disease in ruminants (6). *M. paratuberculosis* is similar to mycobacteria within the *Mycobacterium avium-Mycobacterium intracellulare* group, recently designated the *Mycobacterium avium* complex (20). This is a group of mycobacteria capable of causing a wide range of diseases in animals and humans, primarily as opportunistic pathogens (10), and there are as yet no universally recognized criteria for their precise taxonomic classification (20, 22, 23, 25, 26). In addition, Burnham et al. (4) isolated an organism with the culture characteristics of *Mycobacterium kansasii* from CD tissue. It is therefore apparent that the precise taxanomic classification of CD-isolated mycobacteria is required to establish the precise nature of the organisms isolated from CD tissue.

Many methods of analysis are applicable to the classification of mycobacteria (22, 23, 25). Determination of total sequence homology by measurement of DNA reassociation kinetics has been shown to show good correlation with mycobacterial species classification (2, 14). This method is not sufficiently sensitive, however, to distinguish between closely related species, since homologies between 80 and 100% are not readily distinguished. Restriction endonuclease analysis of chromosomal DNA has also been used to differentiate among mycobacteria (9); however, the method requires preparation of high-molecular-weight DNA, which, when digested, yields a large number of DNA fragments that are difficult to differentiate and interpret.

We have determined the total DNA base sequence homology between the CD-isolated unclassified *Mycobacterium* sp., strain Ben, and the mycobacterial species *M. paratuberculosis*, *M. avium* complex serovars 2 and 5, *M. kansasii*, and *M. phlei* by measurement of DNA reassociation kinetics (19a). This study was capable of distinguishing between the CD isolate and *M. kansasii* and *M. phlei*, but the DNA homology found between the CD isolate and *M. paratuberculosis*, *M. avium* complex serovar 2, and *M. avium* complex serovar 5 was in each case greater than 90% and was indistinguishable from that obtained with homologous DNA. For this reason, we have developed a technique for distinguishing between these closely related mycobacterial species.

The present study describes the cloning of the genome of the unclassified mycobacterium: *Mycobacterium* sp. strain Ben, isolated from CD tissue by Chiodini (7, 8). Cloned DNA probes were used to identify restriction fragment length polymorphisms (RFLPs) in the DNA samples under study to determine the relationship between this organism and other mycobacteria. This method is compatible with standard DNA preparations and produces unambiguous data that clearly distinguish between closely related organisms in a single experiment. Additionally, by the use of a number of probes, accurate values of frequency of DNA base substitution between closely related mycobacteria may be obtained.

MATERIALS AND METHODS

Mycobacterial strains. The following mycobacteria were examined: CD tissue-isolated unclassified *Mycobacterium* sp. strains Ben, Dominic, and Linda (ATCC 43015) (7, 8); *M.*

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paratuberculosis ATCC 19698; *M. avium* complex serovar 2 (Caddigg 16741 [22]); *M. avium* complex serovar 5 (25546-759 [22]); *M. kansasii* (TMC 1201); and *M. phlei* (National Collection of Industrial Bacteria, United Kingdom, strain 8573). DNA prepared from *Neisseria gonorrhoeae*, *Streptococcus pneumoniae* CL3r2S, and *Escherichia coli* were gifts of S. Kothari, St. Georges Hospital Medical School.

Preparation of mycobacterial DNA. Mycobacteria were grown in 7H9 broth as described previously (7, 8) and harvested by centrifugation at 10,000 × *g* for 20 min. Cells were suspended in extraction buffer (0.0132 M phosphate buffer [pH 6.8] containing 0.05 M EDTA, 0.7% NaCl, and 2 mg of lysozyme per ml) and lysed by freeze-fracture in a Hughes Press (17) at −80°C and 15,000 to 25,000 lb/in^2. DNA was extracted by multiple phenol-chloroform extractions, ribonuclease A digestion, and ethanol precipitation (19).

Cloning and manipulation of DNA. Mycobacterial DNA was digested with the restriction endonuclease *Bam*HI and ligated to *Bam*HI-digested and phosphatase-treated plasmid pGEM-1 (Promega Biotec, Madison, Wis.). The ligated DNA was used to transform *E. coli* DH1 cells. Mycobacterial DNA was radiolabeled with ^{32}P by in situ hybridization to bacterial colonies (15).

Standard procedures were used for preparation of plasmid DNA, labeling of DNA, restriction endonuclease digestions, and Southern blotting (18), except that Hybond-N (Amersham U.K., Little Chalfont, United Kingdom) was used as the hybridization membrane. Mycobacterial probes were prepared from recombinant clones by digestion of the cloned DNA with *Bam*HI, electrophoresis and excision of the insert in low-melting-point agarose, and labeling with ^{32}P by second-strand synthesis with the *E. coli* DNA polymerase large fragment, as described previously (12). Hybridization reactions were carried out in 3× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M trisodium citrate [pH 7]) containing 10 mM sodium dihydrogen P_i (pH 7), 0.5% sodium dodecyl sulfate, 0.05% bovine serum albumin, 0.05% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), 0.05% polyvinylpyrrolidone, and 100 μg of denatured salmon sperm DNA per ml at 65°C for 18 h. Filters were washed several times in 1× SSC–0.1% sodium dodecyl sulfate at 65°C and autoradiographed. Hybridized probe was removed by treatment with 0.4 M sodium hydroxide at 45°C for 30 min and neutralized.

Analysis. The banding patterns obtained, when restriction endonuclease digestions of the DNA samples were probed with strain Ben clones, were analyzed by the following two methods.

(i) Presence or absence of a sequence homologous to the cloned probe. The absence of a homologous hybridizing band indicated either that the genomic DNA under test did not contain the probe sequence or that the evolutionary distance between the cloned probe and the test genomic DNA resulted in sufficient divergence that the base homology between the two sequences was too low to allow formation of a stable hybrid under the hybridization conditions used. It has been estimated that for every 1% lowering of homology in a DNA hybrid, the melting temperature (T_m) is lowered by 1.5°C (11). The T_m of strain Ben genomic DNA was measured as 95°C in 1× SSC, and the %G+C was calculated as 66% (19a). With these values, it was estimated (11) that under the hybridization conditions used in these experiments, no signal would have been obtained when the percent homology between the probe and the test DNA sample was less than 74%.

(ii) Analysis of RFLPs. RFLPs are produced by hybridization of the probe to polymorphic restriction fragments in the test DNA. This could be the result of either sequence divergence at the recognition sites for the restriction enzymes (a single base change can lose a site or create a new recognition site) or insertions or deletions of DNA between the sites. Upholt (24) described a quantitative analysis of RFLPs applicable to the analysis of closely homologous DNA (>80% homology) in the absence of DNA rearrangements and when the predominant genetic shift is likely to be a single base change. This analysis has been used to measure sequence divergence among *E. coli* laboratory strains (1) and *E. coli* strains freshly isolated from animals and humans (16). The number of conserved (homologous) fragments in paired restriction endonuclease digests of DNA is determined as a fraction (F) of the total number of hybridizing fragments. From this value is estimated the fraction of substituted bases, P,

$$P \approx 1 - [-F + (F^2 + 8F)^{1/2}/2]^{1/n}$$

where n is the number of bases in the restriction enzyme recognition site. The analysis requires that the fragments produced by restriction enzymes recognizing 4-base sites and 6-base sites be analyzed separately. This can be rearranged to give the fraction of conserved fragments, F, expressed as a function of the fraction of base substitution, P.

$$F = (1 - P)^{2n}/[2 - (1 - P)^n]$$

In many paired digests of highly homologous DNA, no RFLPs are found. To determine the maximum frequency of DNA base substitution between the samples that is compatible with this finding, it was assumed that sampling of the DNA bases follows binomial distribution laws (implied in reference 24). The binomial test statistic Z can be computed from the formula

$$Z = \frac{(P_s - P_m)}{(P_m\, q_m/N)^{1/2}}$$

where P_s is the determined fraction of base substitution. This is zero when no nonhomologous (polymorphic) fragments are found. P_m is the maximum fraction of base substitution, and q_m is the minimum fraction of homologous (unsubstituted) bases. N is the total number of independent bases examined, which is equal to the number of fragments examined times the number of bases in the recognition sequence of the restriction endonuclease producing the fragment pattern. Rearrangement of this equation for $P_s = 0$ gives (since $q_m = 1 - P_m$)

$$P_m = Z^2/(N + Z^2)$$

The value of Z is obtained from tables of area under the normal curve: a level of significance of 95%, used in this study, corresponds to a value of Z of 1.64.

The determined values of the frequency of base substitution will only strictly apply to the region of DNA covered by the probes used. This will, however, be an accurate reflection of total DNA sequence homology if the base substitutions are randomly dispersed throughout the genome. A measure of the truth of this approximation may be obtained from a comparison of the estimates of base substitution rates obtained with individual clones.

RESULTS

A total of 20,000 recombinant clones were obtained from 50 ng of *Mycobacterium* sp. strain Ben DNA. The clones were shown to contain plasmids with inserts of between 100 and >10,000 base pairs. The mean size of the insert was 1,400 base pairs, and since the genome size of strain Ben was estimated as being 3.1×10^6 base pairs by measurement of DNA renaturation kinetics (19a), the entire genome should be represented (with 95% probability) in approximately 15,000 clones (18).

One-thousand clones were transferred to nylon membrane (Hybond N) and were screened in situ with ^{32}P-labeled probe prepared by nick translation of mycobacterial DNA. Approximately 5% of clones were found to give a detectable signal when hybridized to probe prepared from either the CD isolate strain Ben, *M. paratuberculosis*, *M. avium* complex serovar 2, or *M. avium* complex serovar 5, but less than 1% of clones gave a detectable signal when hybridized to either *M. kansasii* or *M. phlei* DNA (data not shown). Hybridizing clones are likely to contain sequences present at high copy number in the strain Ben genome. Clones that showed possible differential hybridization between the mycobacterial DNA probes were selected for further study.

Each mycobacterial DNA (except strain Linda, since only very limited amounts of DNA from this isolate were available) and control samples of DNA isolated from *N. gonorrhoeae*, *S. pneumoniae*, *E. coli*, and humans were digested with *Bam*HI, electrophoresed, and blotted onto hybridization filters. Radiolabeled probes were prepared from strain Ben clones by digestion of plasmid DNA with *Bam*HI, isolation of the recombinant insert by electrophoresis in low-melting-point agarose, and preparation of high-specific-activity ^{32}P-labeled transcripts with *E. coli* DNA polymerase large fragment. Blots of mycobacterial DNA were hybridized to probes prepared from 10 strain Ben recombinant clones. None of the strain Ben probes were found to hybridize to the human or the *S. pneumonia* DNA, although we observed hybridization, shown to be due to the vector sequences in the probes, to *E. coli* DNA and to *N. gonorrhoea* DNA, presumably owing to the presence of a plasmid in these DNA samples (data not shown).

Considering initially only the intensity of signal produced and not the fragment pattern [Materials and Methods, analysis section (i)], cloned probes were found to hybridize to all mycobacteria tested, although not with an equal intensity (two clones); to all mycobacteria tested except (or only very weakly to) *M. phlei* (three clones); to all mycobacteria tested except (or only very weakly to) *M. phlei* and *M. kansasii* (four clones; example in Fig. 1A); to *M. paratuberculosis* and the CD isolates but more weakly to *M. avium* complex serovars 2 and 5. Only one clone (pMB22) was found to give this pattern. This clone hybridized to multiple bands in *M. paratuberculosis* and the CD isolates but to only a single band in *M. avium* complex serovars 2 and 5 (Fig. 1B). All other probes tested gave similar strength of hybridization signal among the CD isolates, *M. paratuberculosis*, and *M. avium* complex serovars 2 and 5, suggesting that most genomic DNA is greater than 74% homologous among these mycobacteria.

The results were then analyzed for the presence of RFLPs. These were almost invariably found, distinguishing between the CD isolates, *M. paratuberculosis*, and *M. avium* complex serovars 2 and 5, as a group, and *M. kansasii* and *M. phlei*. Only one clone (pMB12; Fig. 1A) revealed an RFLP that distinguished between the CD isolates and *M. paratuberculosis* on the one hand and *M. avium* complex serovars 2 and 5 on the other. The clone pMB22 also distinguished between these mycobacteria (Fig. 1B) but could not be analyzed as a simple polymorphism.

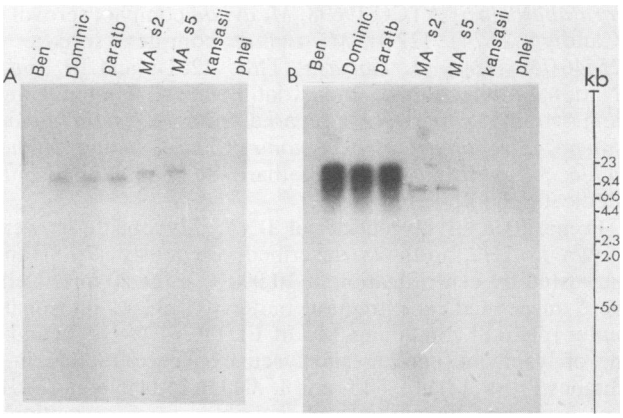

FIG. 1. Hybridization of ^{32}P-labeled strain Ben recombinant plasmid probes to *Bam*HI restriction endonuclease digests of DNA prepared from CD tissue-isolated mycobacteria (strains Ben and Dominic) and classified mycobacteria. Digests (0.1 μg each) were subjected to electrophoresis through 1% agarose and blotted onto a Hybond-N membrane filter. (A) The filter was then hybridized to ^{32}P-labeled strain Ben-cloned probe pMB12 and autoradiographed. (B) The probe was removed by treatment with alkali, as described in the text, and the filter was then hybridized to ^{32}P-labeled strain Ben-cloned probe pMB22 and autoradiographed. Abbreviations: paratb, *M. paratuberculosis*; MA s2, *M. avium* complex serovar 2; MA s5, *M. avium* complex serovar 5.

To identify RFLPs that would further differentiate the DNA samples, DNA from strain Ben, *M. paratuberculosis*, and *M. avium* complex serovar 2 was digested with 11 different restriction endonucleases, and the DNA filters were hybridized to probes prepared from seven recombinant clones. An example of part of such an experiment is shown in Fig. 2. Many RFLPs were detected that differentiated between *M. paratuberculosis* and *M. avium* complex serovar 2, as shown in the *Hin*fI digests in Fig. 2. However, strain Ben DNA invariably gave an identical banding pattern to that obtained with *M. paratuberculosis* DNA. All mycobacterial DNA samples were then analyzed with eight of the combinations of restriction enzymes and the probes that produced RFLPs that distinguished between *M. paratuberculosis* and *M. avium* complex serovar 2. Each CD isolate gave an identical banding pattern to that obtained with *M. paratuberculosis*. *M. avium* complex serovar 5 invariably produced the same banding pattern as *M. avium* complex serovar 2. Examples of two such RFLPs are shown in Fig. 3.

No RFLPs were detected that distinguished between strain Ben and *M. paratuberculosis*. Additionally, no RFLPs were detected that differentiated among the CD mycobacterial isolates (strains Ben, Dominic, and Linda). *M. avium* complex serovars 2 and 5 were similarly indistinguishable by the combinations of restriction enzymes and probes used (for example, Fig. 3).

A quantitative analysis of RFLPs between the DNAs of *M. paratuberculosis* and *M. avium* complex serovar 2 was performed by the method of Upholt (24), as described in Materials and Methods [analysis section (ii)]. The percentage of base substitution was determined for each probe (Table 1), and this was averaged (weighted for the number of

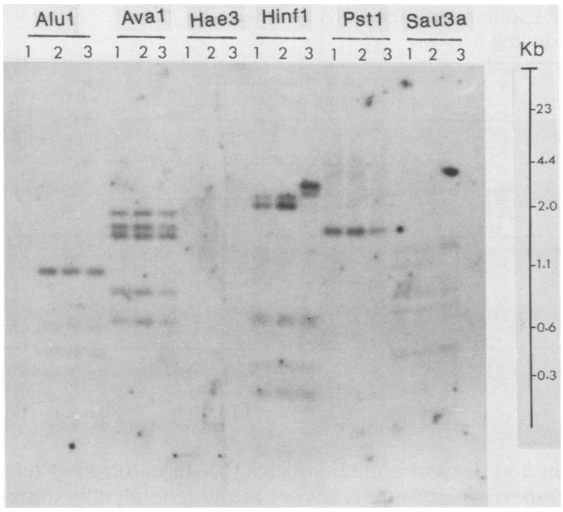

FIG. 2. Hybridization of ^{32}P-labeled strain Ben recombinant probe pMB12 to *Pvu*II restriction endonuclease digests of mycobacterial DNA (0.1 μg). DNA samples were prepared from CD tissue-isolated mycobacterium strain Ben (lane 1), *M. paratuberculosis* (lane 2), and *M. avium* complex serovar 2 (lane 3). Samples were electrophoresed through 1.4% agarose, blotted, hybridized to probe, and autoradiographed as described in the text.

bases examined with each probe) to give a mean value of 0.87% for the base substitution rate. The percentage of base substitution measured between *M. paratuberculosis* and *M. avium* complex serovar 2 varied only from 0.0 to 2.32% with the clones investigated, indicating that the base substitutions were evenly distributed among the segments of DNA probed by the individual clones. A quantitative value for frequency of base substitution between *M. paratuberculosis* and either

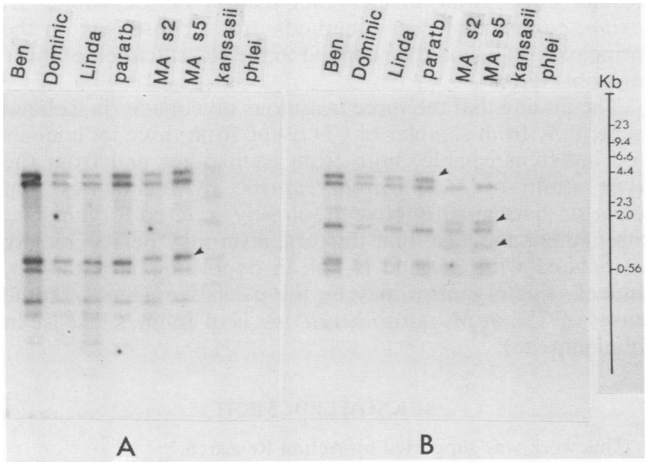

FIG. 3. Hybridization of ^{32}P-labeled CD tissue-isolated *Mycobacterium* strain Ben recombinant probes to restriction endonuclease *Pvu*II digests of DNA prepared from CD tissue-isolated mycobacteria (strains Ben, Dominic, and Linda) and classified mycobacteria. Samples (0.1 μg) were electrophoresed through 1% agarose, blotted, hybridized, and autoradiographed. (A) Probes used were pMB19. (B) The probe was removed, and the filter was hybridized with probe pMB17. Polymorphic bands are marked with arrowheads. Abbreviations: paratb, *M. paratuberculosis*; MA s2, *M. avium* complex serovar 2; MA s5, *M. avium* complex serovar 5.

TABLE 1. Fraction of conserved fragments and inferred frequency of base substitution between *M. paratuberculosis* and *M. avium* complex serovar 2

Probe	Restriction endonucleases recognizing 4-base sites[a]			Restriction endonucleases recognizing 6-base sites[b]		
	No. of fragments examined[c]	F^d	Inferred $\%P^d$	No. of fragments examined	F	Inferred $\%P$
pMB7	27	0.888	1.02	43	0.977	0.23
pMB12	39	0.820	1.72	50	0.960	0.43
pMB16	12	1.000	0.00	22	1.000	0.00
pMB17	34	0.823	2.32	49	0.898	0.71
pMB19	29	0.896	1.04	75	0.853	0.94
pMB20	16	1.000	0.00	64	0.781	1.43
pMB21	16	1.000	0.00	25	0.960	0.84

[a] Enzymes used were *Taq*I, *Alu*I, *Hin*fI, and *Hae*III.
[b] Enzymes used were *Pst*I, *Ava*I, *Sst*I, *Pvu*I, *Pvu*II, *Eco*RI, and *Bst*I.
[c] Obtained by counting total number of analyzable fragments in each digest.
[d] F and P are defined in Materials and Methods. Weighted average value of P = 0.87%; weighted standard error = 0.6%.

M. kansasii or *M. phlei* could not be obtained by this method since DNA rearrangements were frequently found.

For the groups of mycobacteria between which no RFLPs could be found, the number of conserved restriction fragments (RFLPs) that would be expected to occur if the frequency of base substitution between paired DNA samples was either 5 or 1% [Materials and Methods, analysis section (ii)] is presented in Table 2. The maximum frequency of base substitution between the indistinguishable DNA samples was also evaluated and is presented in Table 2 with confidence limits of 95%. For example, if the actual frequency of base substitution between strain Ben and *M. paratuberculosis* was either 5 or 1%, then the number of expected conserved fragments in the 365 fragments examined (123 + 242) would have been 172 (103 + 69) or 311 (109 + 202), respectively, and therefore the expected number of polymorphic bands would have been 193 or 54, respectively. Since no polymorphisms were found, the maximum frequency of base substitution compatible with this finding was calculated as 0.15% with 95% confidence limits.

DISCUSSION

Previous characterization of the CD-isolated mycobacteria has shown them to be mycobactin-dependent organisms with culture characteristics similar to those of *M. paratuberculosis* (8). However, *M. paratuberculosis* is closely related to mycobacteria of the *M. avium* complex, and there is at present no universally recognized criterion for distinguishing among the various organisms in these groups (20, 22, 23, 25). The results described in the present study have shown first, from consideration only of the intensity of hybridization of strain Ben clones to mycobacterial DNA (Fig. 1), that the strain Ben clones were homologous to sequences present in the other mycobacteria tested, in the following order of homology: strain Ben, strain Linda, strain Dominic, *M. paratuberculosis* > *M. avium* complex serovars 2 and 5 > *M. kansasii* > *M. phlei*. *M. avium* complex serovars 2 and 5 were clearly very closely related to *M. paratuberculosis* and strain Ben but could be differentiated from these strains by the hybridization signal produced by one of the clones tested (pMB22; Fig. 1B), which gave multiple bands with *M. paratuberculosis* and the CD isolates but only a single band with *M. avium* complex serovars 2 and 5. Sequences contained in this clone must be absent from

TABLE 2. Estimated maximum frequency of base substitution between DNA samples with identical restriction fragment banding patterns

Mycobacterial DNA compared	Restriction endonucleases recognizing 4-base sites[a]			Restriction endonucleases recognizing 6-base sites[b]			P_m[c] with 95% confidence (%)
	No. of fragments examined	Expected no. of conserved fragments for P[c] of:		No. of fragments examined	Expected no. of conserved fragments for P of:		
		5%	1%		5%	1%	
M. avium serovars 2 and 5	19	11	17	32	14	27	1
Strains Dominic, Linda, and Ben and *M. paratuberculosis*	31	17	27	22	9	18	1
Strain Ben and *M. paratuberculosis*	123	69	109	242	103	202	0.15

[a] For endonucleases, see Table 1, footnote *a*.
[b] For endonucleases, see Table 1, footnote *b*.
[c] P and P_m are defined in Materials and Methods.

both *M. avium* complex serovars 2 and 5; alternatively, the sequences must have diverged so that the DNA homology is less than 74%. Each of the CD isolates gave hybridization signals indistinguishable from those obtained with *M. paratuberculosis*.

Furthermore, many RFLPs were found that could clearly distinguish between the American type strain of *M. paratuberculosis* and both *M. avium* complex serovars 2 and 5 (Fig. 1A, 2, and 3). All three CD-isolated mycobacteria were found to be indistinguishable from the type strain of *M. paratuberculosis* but clearly distinguishable from *M. avium* complex serovars 2 and 5, with each RFLP investigated. The absence of DNA polymorphisms and the low maximum frequency of base substitutions between the CD isolates and *M. paratuberculosis* (<0.15% for strain Ben-*M. paratuberculosis*) is very strong evidence that they are the same species. It is possible, however, that small differences in DNA content, such as possession of a plasmid or bacteriophage, could alter the biological properties of the organisms associated with CD, resulting in differing pathogenicities for animals and humans.

The frequency of base substitution between *M. paratuberculosis*, strain Ben, and *M. avium* complex serovar 2 was calculated as 0.87 ± 1.2%, implying that the DNA homology among these organisms is 99.13 ± 1.2%. This high value for DNA homology might suggest that *M. paratuberculosis* and *M. avium* complex serovar 2 are in fact the same species, particularly if a value of >90% DNA homology is taken as indicative of this, as suggested by Baess (2). However, the present study demonstrated several RFLPs distinguishing between the type strain of *M. paratuberculosis* and *M. avium* complex serovar 2, and in each case, the CD-isolated organisms gave the *M. paratuberculosis* pattern and *M. avium* complex serovar 5 gave the *M. avium* complex serovar 2 pattern. The conservation and genetic linkage of RFLPs between *M. paratuberculosis* and the CD-isolated organisms on the one hand and *M. avium* complex serovars 2 and 5 on the other suggests that they belong to two separately evolving groups.

It must be recognized that the use of mean values of DNA homology to identify the species has limitations. A high proportion of base substitutions in third-base positions of amino acid codons could produce organisms with reduced base sequence homology but identical biological properties. Conversely, small base changes in critical genes could drastically modify the biological properties of an organism while retaining very high base homology with the parent organism. *M. avium* is an environmental opportunistic organism, while *M. paratuberculosis* is a well-recognized pathogen in a variety of animal species (6). In addition, while *M. paratuberculosis* and *M. avium* are biochemically similar, a variety of biochemical tests reveal differences in activity (5). The small amount of sequence divergence found between these organisms may account for these differences; the genetic change could be in the form of DNA mutation or could involve the loss or gain of genetic material (plasmids, bacteriophages, insertional elements). The question of whether *M. paratuberculosis* should be considered the same species as organisms of the *M. avium* complex must await a more thorough examination of the whole complex and further strains of *M. paratuberculosis*.

No polymorphisms were found that distinguished between *M. avium* complex serovars 2 and 5. The maximum frequency of base substitution between these organisms was evaluated as being less than 1%, implying a DNA homology of greater than 99%. This is consistent with the results of Baess (3), who obtained a value of 92% DNA homology for these organisms by using solution hybridization and concluded they were the same species. Also, catalase characterization by Wayne and Diaz (26) have indicated that the strains are the same species. Again, the significance of these results must await further examination of the whole *M. avium* complex by these methods and a consensus on the principles that should be applied to the classification of these mycobacteria.

The finding that the three fastidious mycobacteria isolated seperately from samples of CD tissue from three individuals are indistinguishable, both from each other and from the type strain of *M. paratuberculosis*, by techniques that clearly distinguish between closely related organisms, strengthens the case that this organism may be specifically associated with CD and is not an opportunistic pathogen. Indeed, this organism may be the causative agent in some cases of CD, as *M. paratuberculosis* is of Johne's disease in ruminants (6).

ACKNOWLEDGMENT

This work was supported by Action Research.

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